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# Anaerobic Oxidation of Methane in Sediments of Lake Constance, an Oligotrophic Freshwater Lake[▽]

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**Anaerobic oxidation of methane (AOM) with sulfate as terminal electron acceptor has been reported for various environments, including freshwater habitats, and also, nitrate and nitrite were recently shown to act as electron acceptors for methane oxidation in eutrophic freshwater habitats. Radiotracer experiments with sediment material of Lake Constance, an oligotrophic freshwater lake, were performed to follow $^{14}CO_2$ formation from $^{14}CH_4$ in sediment incubations in the presence of different electron acceptors, namely, nitrate, nitrite, sulfate, or oxygen. Whereas $^{14}CO_2$ formation without and with sulfate addition was negligible, addition of nitrate increased $^{14}CO_2$ formation significantly, suggesting that AOM could be coupled to denitrification. Nonetheless, denitrification-dependent AOM rates remained at least 1 order of magnitude lower than rates of aerobic methane oxidation. Using molecular techniques, putative denitrifying methanotrophs belonging to the NC10 phylum were detected on the basis of the *pmoA* and 16S rRNA gene sequences. These findings show that sulfate-dependent AOM was insignificant in Lake constant sediments. However, AOM can also be coupled to denitrification in this oligotrophic freshwater habitat, providing first indications that this might be a widespread process that plays an important role in mitigating methane emissions.**

Freshwater lakes account for 2 to 10% of the total emissions of the potent greenhouse gas methane (1) and are therefore an important part of the global methane cycle (24, 56). The major part of methane is formed biologically by methanogenic archaea in anoxic environments, where alternative electron acceptors are lacking (8). Some methane is lost from the sediments due to ebullition or mixing events (5, 9), but most of it is readily oxidized by aerobic methanotrophic bacteria when they reach the oxic biosphere (17). Aerobic methanotrophs activate methane using molecular oxygen in a monooxygenase reaction to cleave the strong C—H bond (28). Anaerobic oxidation of methane (AOM) with sulfate as electron acceptor is carried out by methanogen-like archaea, so-called anaerobic methanotrophic (ANME) archaea, in syntrophic cooperation with sulfate-reducing bacteria (3, 19, 20, 57). Although no defined coculture is available to date (37, 38), metagenomic analysis (16, 33) and the discovery of an abundant, methyl coenzyme M reductase-like protein in microbial mats catalyzing AOM (32) provided indications that sulfate-dependent AOM in all probability operates as a reversal of methanogenesis. The energy gain (according to the change in the Gibbs free energy [$\Delta G°'$]) in sulfate-dependent AOM according to equation 1 is close to the theoretical minimum for ATP synthesis ($\Delta G°' = -20$ kJ mol$^{-1}$) (45), which could hardly feed two organisms in a syntrophic cooperation.

$$CH_4 + SO_4^{2-} + H^+ \rightarrow CO_2 + HS^- + 2H_2O,$$
$$\Delta G°' = -21.3 \text{ kJ mol}^{-1} \text{ CH}_4 \quad (1)$$

Therefore, this process is preferentially observed in marine environments at >800-m water depths and under high methane pressures. AOM coupled to iron and manganese reduction (2) or humic compound reduction (47) has been reported recently, but a direct coupling of these electron acceptors to AOM was not shown, and the organisms responsible for these processes are unknown. However, the energy yield of AOM coupled to those proposed electron acceptors would be substantially higher than that with sulfate, allowing the reactions to take place at lower substrate concentrations (52). AOM can also be coupled to denitrification according to equation 2 (41):

$$3CH_4 + 8NO_2^- + 8H^+ \rightarrow 3CO_2 + 4N_2 + 10H_2O,$$
$$\Delta G°' = -928 \text{ kJ mol}^{-1} \text{ CH}_4 \quad (2)$$

This process does not depend on a syntrophic cooperation with archaea (13) but is carried out by bacteria affiliated with the NC10 phylum, a phylum without any cultured representatives so far. Few enrichment cultures of this type have been obtained to date (14, 23, 41), and a metagenome was assembled from two enrichments. It turned out that the denitrifying NC10 bacteria produce oxygen from nitrite via NO (12). Thus, this type of methane oxidation takes place in anoxic environments, but the chemically challenging activation of methane does not proceed anaerobically, and methane is activated through a methane monooxygenase reaction as in aerobic methanotrophs. A gene cluster encoding particulate methane monooxygenase has been identified in the metagenome and is actively transcribed and translated (12).

Sulfate-dependent AOM was reported mainly for marine environments (3, 30, 53), and there is little evidence for AOM in freshwater habitats, where it may often be masked by aerobic methane oxidation due to the close spatial proximity of the reactant transition zones (49). Sulfate-dependent AOM was reported for Lake Plußsee, a eutrophic lake (11), rice paddies (35), peat lands (47), and landfills (15). AOM coupled

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TABLE 1. Sampling locations, sampling dates, and estimated maximum initial methane oxidation rates of Lake Constance sediment incubations

| Habitat (depth) | Abbreviation | Sampling date (day.mo.yr) | Maximum initial methane oxidation rate[b] | | |
|---|---|---|---|---|---|
| | | | No addition | Nitrate | Ambient air |
| Profundal (ca. 80 m) | prof1 | 17.02.2009 | NA[a] | 3.6 | 27 |
| Profundal (ca. 80 m) | prof2 | 06.10.2009 | NA | 2.7 | 38 |
| Profundal (ca. 120 m) | prof3 | 09.02.2010 | NA | 1.8 | 44 |
| Littoral (2–3 m) | litt1 | 16.11.2009 | NA | NA | 18 |
| Littoral (2–3 m) | litt2 | 14.01.2010 | 0.08 | 0.6 | 63 |

[a] NA, rates were not calculated if the $^{14}CO_2$ formation was within the background scatter of the values at time zero and values for sterile controls. With sulfate as electron acceptor, the $^{14}CO_2$ formation was within the background scatter in all experiments.

[b] With the given electron acceptors, in nmol $d^{-1}$ (ml sediment)$^{-1}$.

to denitrification was reported for nutrient-rich habitats, such as contaminated groundwater (48) and sewage sludge (25). Enrichments were obtained from eutrophic canals and ditches (14) and a mixed inoculum (23), but direct measurements of denitrification-dependent AOM in freshwater samples are lacking. Some indications on the distribution of this process might be derived from 16S rRNA gene sequences affiliated with NC10 bacteria which have been obtained from various freshwater habitats (14). However, hardly any information is available on the distribution of methanotrophy in this uncultured phylum, and the presence of 16S rRNA gene sequences is not a solid indication for the presence of this process.

In Lake Constance, an oligotrophic freshwater lake, concentration profiles of methane and oxygen indicated that methane might also be oxidized anaerobically in Lake Constance sediments (43), but microaerobic methane oxidation and temporal disturbances of the gradients could not be excluded. In the present study, we checked for AOM in the sediments of this lake; tested sulfate, nitrate, and nitrite as possible electron acceptors for AOM; and searched for the responsible microorganisms by molecular methods.

## MATERIALS AND METHODS

**Sediment sampling.** Littoral sediment samples were collected with a sediment corer (51) with plastic tubes of 80 mm inner diameter from the lower infralittoral zone (Litoralgarten; 47°41′N, 9°12′E) of Lake Constance at a water depth of 2 to 3 m (Table 1). Profundal sediments were collected with a ship-born multicorer with the same plastic tubes from a depth of 80 to 120 m in upper Lake Constance, between Wallhausen and Egg, Germany (Table 1). The profundal core used for the construction of the clone library was sampled in front of the Isle of Mainau (47°42′N, 9°12′E). All sediment cores were at least 20 cm long. The lower end of the core was closed with a plug without trapping of gas bubbles, and the upper part was capped with a screw-cap lid, avoiding trapping of a gas bubble in the overlying water to prevent resuspension of the sediment during transport. The closed sediment cores were transported to the laboratory with avoidance of percussions. Thus, the investigated sediment layer of 1- to 4-cm sediment depth was undisturbed until the core was cut in the anoxic tent, where the possibility of oxygen contamination of the investigated sediment layer could be excluded. All sediment cores for radiotracer experiments were collected between February 2009 and February 2010 and immediately stored at 4°C, and experiments were started within 24 h after sampling.

**Preparation of $^{14}CH_4$.** A culture of *Methanospirillum hungatei* was grown in freshwater medium as described previously (54), with some modifications (34), but 20 mM HEPES buffer (pH 7.2) was used instead of bicarbonate buffer. $H_2$-$CO_2$ (80:20) was added to an overpressure of 0.5 bar. After growth was visible, nitrogen was bubbled through the culture to remove remnant $CO_2$. A 1:5 (vol/vol) mix of $^{14}CO_2$ and $H_2$ was added, and nitrogen was supplied further to an overpressure of 0.5 bar. After 1 week, premixed $H_2$-$CO_2$ (80:20) was added to an overpressure of 0.5 bar. One week later the gas phase was removed by simultaneously adding medium. The gas phase was transferred into a 20-ml serum bottle filled with 1 M NaOH in dithionite-reduced freshwater medium containing 3 M NaCl (to decrease gas solubility in the liquid phase), while simultaneously, some of the liquid phase was removed to release overpressure. The gas phase was then taken out with a syringe that contained hopcalite to remove traces of $^{14}CO$ (18) and injected again into a 20-ml serum bottle as described before to trap $^{14}CO_2$. After an additional transfer, the tracer gas was stored until further use. All transfers were carried out with pregassed ($N_2$ or He) one-way plastic syringes with a fitted luer-lock Teflon valve. Resazurin was added as a redox indicator in all liquid phases.

**Radiotracer experiments.** Sediment cores were introduced into an anoxic tent, and the uppermost 1 cm was removed to omit the oxic sediment layers from the experiment. Sediments from 1- to 4-cm depths of 1 to 3 sediment cores of the same location and sampling date were mixed and diluted with a few ml (at maximum, 1/10 of sediment volume) of freshwater medium (54) to obtain a soft, viscous sediment slurry that could be transferred by a cutoff 3-ml or 5-ml plastic syringe. The slurry was split into different treatments, and the desired electron acceptor (2 mM $NaNO_3$, 1.5 mM $NaNO_2$, or 2 mM $NaSO_4$) was added. Stock solutions were freshly prepared with double-distilled water, filter sterilized, and degassed by repeated vacuum/$N_2$ treatment, and sodium dithionite was added to secure anoxia. Three milliliters of the treated sediment was transferred with a cutoff plastic syringe into 9-ml serum bottles, closed with black butyl rubber stoppers, and capped with aluminum crimp caps. The gas phase was then flushed with pure $N_2$ to remove the hydrogen present in the anoxic tent, and afterwards the tracer was added in a glove box gassed with $N_2$. The tracers were diluted with pure nonlabeled methane to allow the addition of methane to an equivalent of 10 μmol per liter slurry. Specific activities of the injected tracers were $1 \times 10^6$ to $2 \times 10^6$ dpm. All samples were incubated in an $N_2$-flushed plastic container at 4°C to mimic the *in situ* temperatures. After incubation, samples were alkalinized with NaOH (0.5 M final concentration) and stored overnight at room temperature. Single vials for every measurement were used to avoid false-positive results due to oxygen contamination during sampling. One milliliter of the gas phase was removed with a one-way plastic syringe with a fitted luer-lock Teflon valve for $^{14}CH_4$ radioactivity and $CH_4$ concentration measurements. Samples were bubbled with $N_2$ for 5 min to remove remaining $^{14}CH_4$, and the vial was connected via tubes and needles to three 5-ml scintillation vials filled with 2 ml of Carbosorb E absorber (Perkin Elmer) in series as described previously (58). The tightness of the system was checked each time with soapy water and by injecting nitrogen gas into the vial before trapping of $CO_2$ started. The slurry was acidified with 37% HCl until no gas formation was visible anymore and was bubbled afterwards with nitrogen to flush remaining $^{14}CO_2$ into the trapping solution. An equal volume of scintillation cocktail Permafluor E+ (Perkin Elmer) was added, the components were mixed, and the vial was stored overnight in the dark to reduce luminescence. Samples were analyzed in an LS 6100IC scintillation counter (Beckman). Initial maximum methane oxidation rates were estimated from the increase of $CO_2$ between start values and the highest observed values of $^{14}CO_2$ in the first days of the experiments. The effects of nitrate, ambient air, and no additional electron acceptor on methane oxidation were tested in all experiments, the effect of nitrite amendment was tested in experiment prof2 and both littoral sediments, and the effect of sulfate addition was investigated in experiments prof1 and prof2 and both littoral sediments.

Samples of the gas phase used to determine the radioactivity of $^{14}CH_4$ were transferred into a 9-ml serum bottle filled with 7 ml toluene, incubated overnight at room temperature, transferred into a scintillation vial containing 10 ml LumaSafe Plus scintillation cocktail (Perkin Elmer), and analyzed as described before. Solubility of methane in toluene was calculated after published values (59). The other sample was transferred into a 9-ml serum bottle containing 3 ml saturated salt solution and stored upside down at −20°C until the methane concentration was determined. Methane was determined using a 6000 Vega series 2 gas chromatograph (Carlo Erba Instruments) as described previously (40). Nitrate and nitrite were estimated with Merckoquant test strips (Merck) to estimate the time when nitrate and nitrite had disappeared completely.

**Molecular detection of NC10 bacteria.** DNA was extracted from two 500-mg (fresh weight) sediment samples with a NucleoSpin soil kit (Macherey-Nagel) using a BioSavant fast prep instrument (Bio 101) according to the manufacturers' instructions. The DNA concentration was measured photometrically at 260 nm using a BioPhotometer (Eppendorf). PCR was carried out using the NC10-specific primers 202F and 1043R as published previously (14), but after analysis of published NC10 sequences introducing two wobbles (NC10-1043Rdeg, 5′-TCTCCRCGYTCCCTTGCG-3′; NC10-202Fdeg, 5′-RACCAAAGGRGGCGAGCG-3′). After adjustment of the PCR program to 94°C for 1 min, followed by

32 cycles of 1 min at 94°C, 45 s at 67°C, and 90 s at 72°C with a final elongation of 7 min at 72°C, PCR products of proper size were obtained directly from sediment DNA extracts, and only sequences affiliated with the NC10 phylum were obtained. Primers for amplification of the *pmoA* gene were designed manually using a multiple alignment of Lake Constance *pmoA* clones and the one *pmoA* sequence available from NC10 bacteria from the assembled genome of "*Candidatus* Methylomirabilis oxyfera" (GenBank accession no. FP565575.1) (12) with MEGA4 software (50). Two primers were designed: NA638Rdeg (5′-RAATGTTCGRAGCGTVCCBC-3′) and NA720R (5′-TCCCCATCCACA CCCACCAG-3′). These primers amplified only novel NC10-related *pmoA* genes and no known *pmoA* genes of aerobic methanotrophs from our samples. PCR targeting the *pmoA* gene was performed using primer A189f (21), together with one of the newly designed primers and the PCR program described previously (6). Two to 20 ng of extracted DNA was used for all PCRs. Pooled PCR products of at least 3 PCRs were purified with a DNA clean and concentrator kit (Zymo Research).

For construction of clone libraries, the purified DNA was cloned using a TA cloning kit (Genaxxon) according to the manufacturer's instructions. Clones were picked, and after a PCR using the M13 primer pair, the product was sent for sequencing (GATC Biotech, Konstanz, Germany). A total of 42 16S rRNA gene sequences were obtained for profundal sediment and 23 sequences for littoral sediments. Rarefaction analysis of the clone libraries was carried out using aRarefactWin software (version 1.3; S. Holland, Stratigraphy Lab, University of Georgia, Athens, GA; www.uga.edu/~strata/software/), and Chao1 estimators (7) were determined with EstimateS software (version 8.2; R. K. Colwell; http://purl.oclc.org/estimates) for each clone library.

**Phylogenetic analysis.** 16S rRNA gene sequences were aligned using the SINA webaligner program (http://www.arb-silva.de/aligner/), and inferred PmoA sequences were aligned with the ClustalW algorithm implemented in MEGA4. Phylogenetic trees were constructed with MEGA4 software (50). Different tree construction methods were compared and yielded similar results. The shown phylogenetic tree based on 16S rRNA gene sequences was constructed using the minimum evolution method with the pairwise deletion option. Evolutionary distances were computed using the Tajima-Nei method. There were a total of 875 positions in the final data set. The shown tree based on PmoA sequences was constructed using the minimum evolution method with the pairwise deletion option. Evolutionary distances were computed using the JTT matrix-based method, with a total of 191 positions in the final data set (25a). The pairwise deletion option was chosen to allow the inclusion of shorter sequences because not many reference sequences were available. When short sequences were excluded and phylogenetic analysis was performed using the complete deletion option, the same sequences clustered together but some deeper-branching nodes changed, also indicated by low bootstrap values in the final tree (see Fig. 3).

**Nucleotide sequence accession numbers.** Nucleotide sequences were deposited at the National Center for Biotechnology Information under accession numbers HQ906501 to HQ906564 (16S rRNA gene sequences) and HQ906565 to HQ906579 (*pmoA* sequences).

## RESULTS

**Sampling.** All sediment cores showed a defined stratification. Profundal sediment cores had a soft homogeneous yellow-brownish top layer and dark sulfidic fine-grained material at a 3- to 5-cm depth. The littoral cores differed in their compositions. One core (litt1) had a thin soft and beige surface layer of approximately 1 cm and then 0.5 cm of blackish sediment containing parts of mussel shells and consisting of very fine gray material, probably lake marl, below. The other littoral sediment core (litt2) and the core used for molecular work had a 2-cm-thick layer of soft beige material and turned black in the deeper layers. *Chara* spp. grew on the sediment, and parts of mussel shells were visible throughout all investigated sediment layers.

**Anaerobic oxidation of methane in sediment incubations.** The influence of different electron acceptors on AOM was investigated using radiotracer experiments. Three independent experiments were performed with profundal sediments and two with littoral sediments (Table 1). All experiments investigating AOM in profundal sediments yielded similar results.

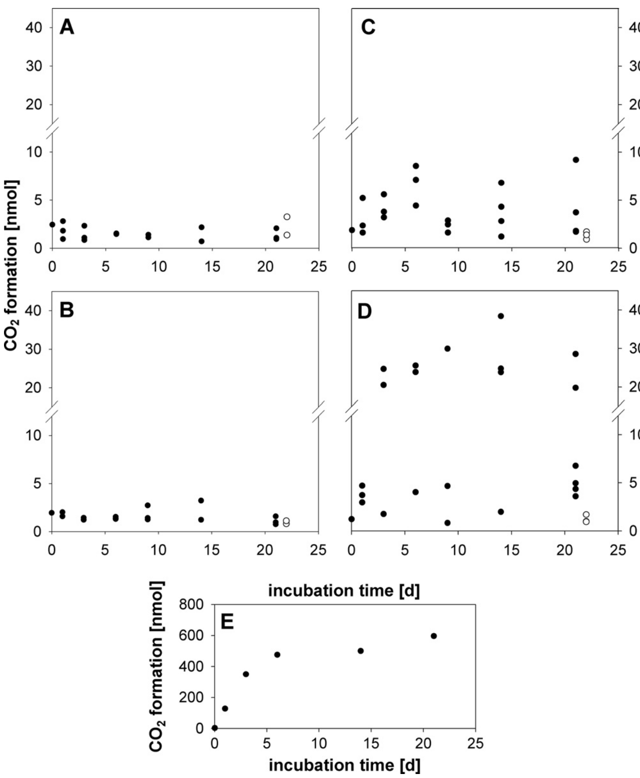


FIG. 1. $CO_2$ formation, calculated from $^{14}CO_2$ formation from $^{14}CH_4$, in incubations of Lake Constance profundal sediment. One representative experiment of three is shown (prof2). The following electron acceptors were added: no added electron acceptor (A), 2 mM sulfate (B), 1.5 mM nitrite (C), 2 mM nitrate (D), and ambient air (E). Filled symbols, sample values; open symbols, sterile controls.

Without addition of an external electron acceptor, $^{14}CO_2$ values remained within the background level in all profundal sediment incubations. Addition of sulfate did not enhance formation of $^{14}CO_2$ in any profundal sediment (Fig. 1). Nitrate caused a clear stimulation of $^{14}CO_2$ formation compared to untreated controls in all profundal sediment incubations (Fig. 1). Estimated AOM rates in nitrate-amended treatments ranged from 1.8 to 3.6 nmol day$^{-1}$ (ml sediment)$^{-1}$ (Table 1). Nitrite addition led to slightly elevated $^{14}CO_2$ values in the profundal sediment tested, but due to high scatter (Fig. 1), no AOM rates were calculated. Control assays under air showed $^{14}CO_2$ formation rates that were about 1 order of magnitude higher than those with nitrate-amended treatments. Results obtained with the two littoral sediment incubations differed in some cases. In the first experiment with littoral sediment (litt1), no enhanced formation of $^{14}CO_2$ was detectable when sulfate, nitrite, or nitrate was added (Fig. 2). The second littoral sediment investigated (litt2) showed enhanced $^{14}CO_2$ formation without any addition, but no AOM was detectable with addition of sulfate or nitrite as electron acceptor (Fig. 2). On the other hand, nitrate addition enhanced $^{14}CO_2$ formation in this littoral sediment, although to a lower extent than in profundal sediments (Table 1). The oxic treatments showed $^{14}CO_2$ formation rates that were 2 orders of magnitude higher than the rates with nitrate treatment in experiment litt2 (Table 1).

Nitrate-dependent AOM stopped in all experiments after a few

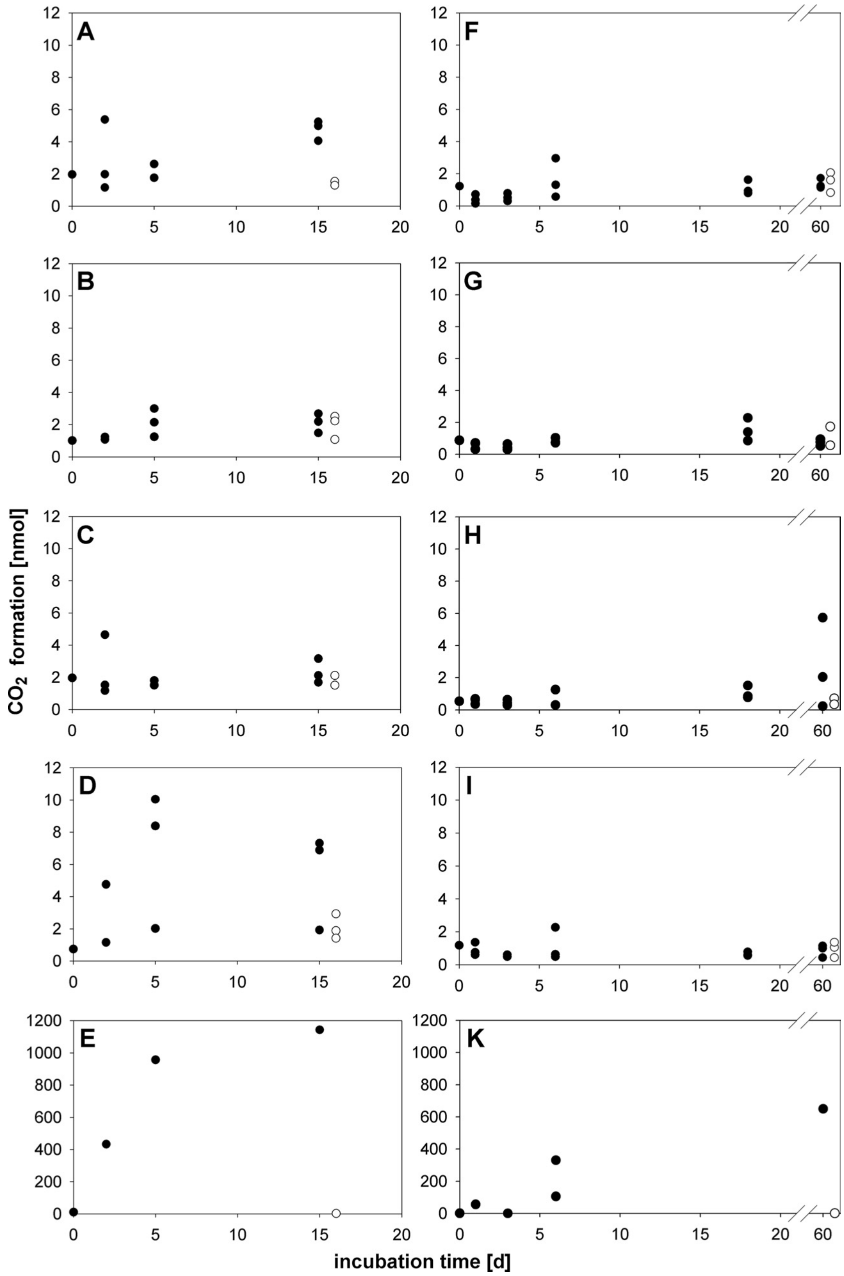


FIG. 2. $CO_2$ formation, calculated from $^{14}CO_2$ formation from $^{14}CH_4$, in incubations of Lake Constance littoral sediment. In experiment littoral 1 (A to E) and experiment littoral 2 (F to K), the following electron acceptors were added: no added electron acceptor (A and F), 2 mM sulfate (B and G), 1.5 mM nitrite (C and H), 2 mM nitrate (D and I), and ambient air (E and K). Filled symbols, sample values; open symbols, sterile controls.

days, which coincided with the time when nitrate was depleted in nonlabeled control vials (usually after 5 days; data not shown). Refeeding nitrate on day 15 in experiment prof2 did not cause a resumption of $^{14}CO_2$ formation. However, even in profundal sediments, nitrate-dependent AOM accounted for less than 5% of the nitrate consumption in the treatments. High $^{14}CO_2$ formation was observed only in about 50% of the replicates even in positive experiments, whereas in the remaining vials, only low or sometimes no $^{14}CO_2$ production was detectable.

Headspace methane concentrations were measured in experiments prof1, prof3, and litt2 and showed no changes over time in any of the anoxic profundal treatments. During incubation of the littoral sediment, however, methane increased from 0.15 μmol to 0.53 μmol and 0.37 μmol without addition and with addition of sulfate, respectively. No changes were observed in the nitrate and nitrite treatments. Methane concentrations decreased in the oxic treatments.

**Diversity of NC10 bacteria.** The 16S rRNA gene of bacteria allocated to the candidate division NC10 (NC10 bacteria) was successfully amplified directly from sediment DNA extracts.

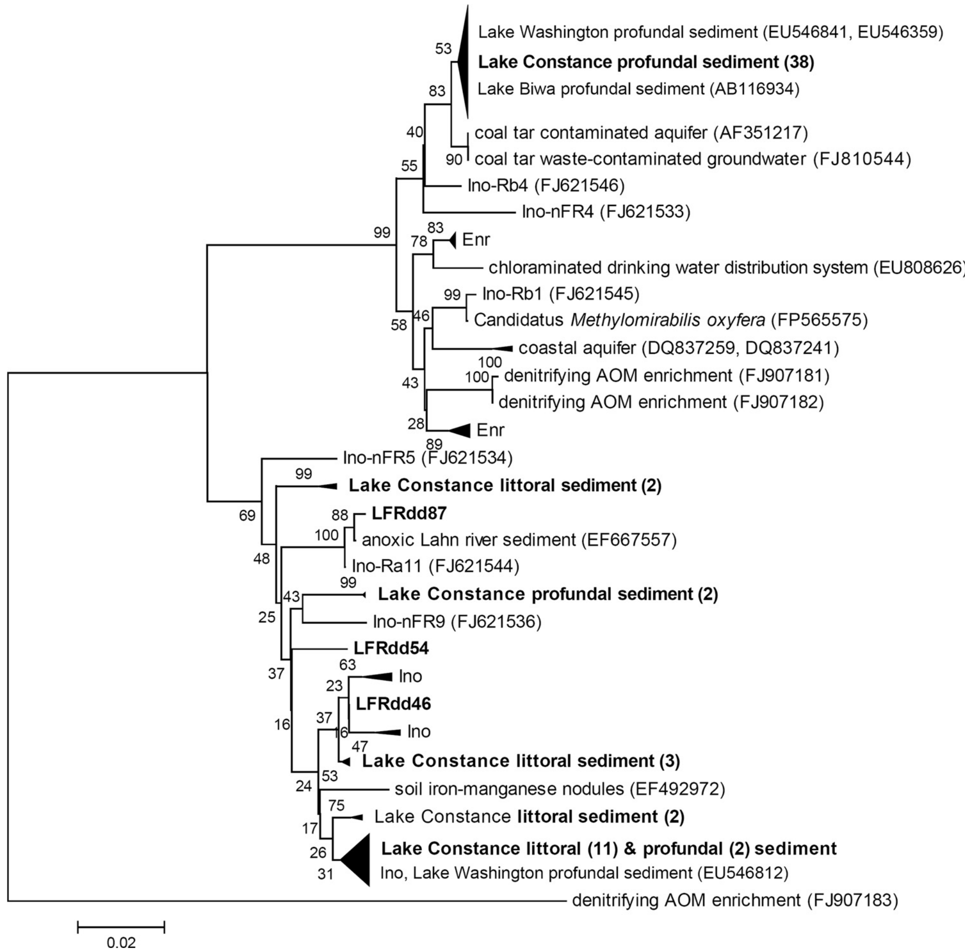


FIG. 3. Phylogenetic tree of the NC10 bacterial 16S rRNA gene sequences obtained from Lake Constance sediments. The tree was constructed using the minimum evolution method choosing the pairwise deletion option. Evolutionary distances were computed using the Tajima-Nei method. Bootstrap values were calculated from 1,000 replicate trees. The scale bar represents the number of substitutions per site. Clones obtained in this study are shown in boldface, and the number of clones or accession numbers are given in parentheses. Ino, sequences from the inoculum of a denitrifying AOM enrichment; Enr, sequences from the enrichment after 6 months (14).

Clone libraries targeting the 16S rRNA gene with the specific primer pair were constructed from littoral and profundal sediments. The clone libraries contained only sequences belonging to the NC10 phylum, thus verifying the specificity of the primers. Clustering of the 16S clones (profundal, $n = 42$ clones; littoral, $n = 23$ clones) to operational taxonomic units (OTUs) using a 3% threshold resulted in 3 OTUs for profundal sediment and 5 OTUs for littoral sediment, with Chao1 richness estimators of $4.81 \pm 1.34$ and $5.25 \pm 0.64$, respectively.

The obtained sequences could be assigned to two main groups of NC10 bacteria, namely, groups A and B, according to Ettwig et al. (14). Clones belonging to group A were obtained only from profundal sediments, whereas clones belonging to group B were obtained from both littoral and profundal sediments. All clones belonging to group A showed a maximum sequence diversity below 1% (average, 0.4%); thus, they likely represent one species that accounts for more than 90% of the profundal clones. Group B clones were more diverse, with 5% maximum sequence diversity (average, 2.2%).

The clones belonging to subgroup A of the NC10 phylum exhibited the highest sequence similarity to Lake Washington and Lake Biwa sediment clones. These groups clustered separately but closely adjacent to the sequences from different denitrifying AOM enrichments and "*Candidatus* Methylomirabilis oxyfera" (Fig. 3). Clones in group B were related to different environmental clones, including the inoculum of a bioreactor and inocula of other freshwater habitats.

**Presence of functional marker gene *pmoA* in Lake Constance sediments.** *pmoA* genes were successfully amplified only from profundal and not from littoral sediments using two newly designed reverse primers. With both primers, only PCR products of the expected size were obtained. Ten sequences were obtained using reverse primer NA638Rdeg, and five were obtained using reverse primer NA720R. All sequences exhibited maximum sequence diversities of 2.5% at the amino acid level and of 1.1% at the nucleotide level. Phylogenetic analysis showed that the sequences cluster closely with the two PmoA sequences known from NC10 bacteria, namely, "*Candidatus* Methylomirabilis oxyfera" strain Twente and strain Ooji (Fig. 4). The sequences obtained from Lake Constance sediments share 3.5% to 5.5% amino acid identity with the sequences of "*Candidatus* Methylomirabilis oxyfera."

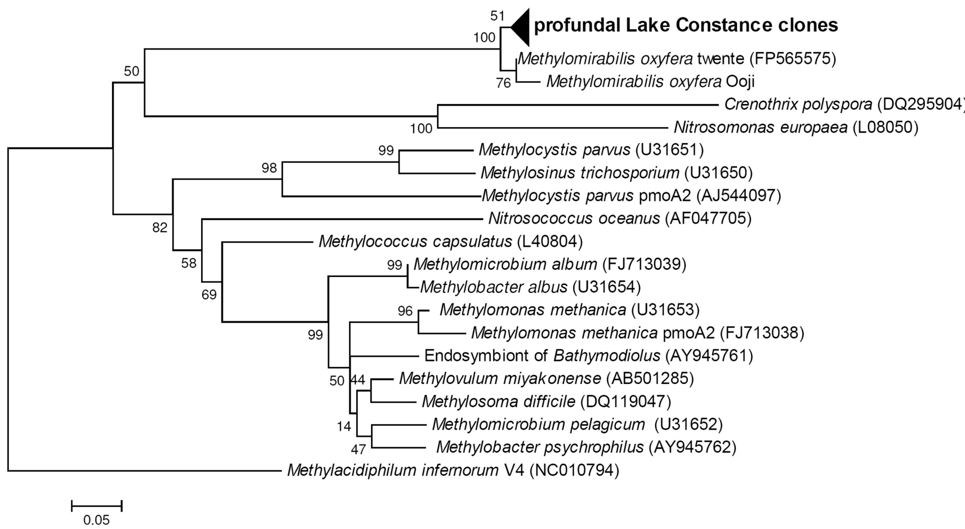


FIG. 4. Phylogenetic tree of the NC10 bacterial PmoA sequences obtained from Lake Constance sediments constructed using the minimum evolution method choosing the pairwise deletion option. Evolutionary distances were computed using the JTT matrix-based method. The scale bar represents the number of substitutions per site. Bootstrap values were calculated from 1,000 replicate trees.

## DISCUSSION

**Anaerobic oxidation of methane in sediment incubations.** AOM was detected in profundal sediments only with nitrate and to a lesser extent with nitrite as electron acceptor. The lower rates with nitrite could be explained by the fact that nitrite at a 1.5 mM concentration might be toxic to NC10 bacteria (22). On the other hand, denitrifying anaerobic methanotrophs have been shown to prefer nitrite over nitrate in enrichment cultures (23, 41). Although these bacteria have the enzymes to use nitrate directly (12), methane oxidation does not yield sufficient reducing equivalents to fuel a completely nitrate-dependent metabolism with molecular oxygen as an intermediate for methane activation. The oxidation of methane to $CO_2$ yields 8 redox equivalents, but due to a monooxygenase reaction, 4 redox equivalents are consumed to reduce $O_2$ to $H_2O$ and the —OH group of methanol. Thus, the remaining 4 redox equivalents are sufficient to reduce 2 nitrite (which consumes 2 redox equivalents) but not to reduce 2 nitrate (which consumes 6 redox equivalents) to $N_2$ and $O_2$. The other 2 redox equivalents could be used for oxygen respiration (55), which would lead to the stoichiometry of nitrite-dependent AOM previously observed (equation 1) (12). In sediments, numerous denitrifying bacteria can provide nitrite from nitrate and denitrification was obvious in our incubations because nitrate disappeared completely, but at a maximum, 5% was consumed by denitrifying AOM. It was already hypothesized that NC10 bacteria may cooperate with unidentified bacteria which reduce nitrate to nitrite or with ammonium-oxidizing bacteria (22, 60).

The high scatter of denitrification-coupled AOM in profundal sediments remains enigmatic, as the sediment appeared very homogeneous and had been mixed well after addition of the electron acceptor. Nonetheless, small differences in sediment composition or unknown factors might influence the competition for nitrate or, subsequently, nitrite between bacteria, thus affecting methane oxidation coupled to denitrification. Littoral sediments were more heterogeneous, and the small volume of 3 ml used in the replicate assays in this study may not be sufficient to produce identical data, considering the size of, e.g., plant roots and small invertebrates, which were likely not distributed evenly among the treatments and might cause significant differences among replicate samples. Furthermore, not much is known about the susceptibility of NC10 bacteria to environmental changes, as the only physiological data come from enrichment cultures that ran continuously for several months before significant AOM rates were detected (14, 23, 41). If NC10 bacteria depend on redox gradients at oxic-anoxic interfaces in their natural environments, as hypothesized by Zhu et al. (60), their activity might be restricted to a few millimeters and conditions in our batch experiments may sustain their activity only for a short period of time. This might also be a reason why nitrate-dependent AOM was found only in one of the two littoral sediment samples. The plants and the thicker surface layer indicate that mechanical disturbances, e.g., by wave action, might be of minor importance at this site, and therefore, the geochemical gradients are more stable, whereas the other sediment was prone to mixing and did not provide a suitable habitat. Beyond this, plant roots are known to establish oxic-anoxic interfaces in sediments (4).

Aerobic methane oxidation rates obtained in our radiotracer experiments are comparable to rates measured before by conventional gas-phase analysis (10). However, substrate limitation caused by slow diffusive transport and the unnaturally high concentrations of the added electron acceptors prohibit exact calculations of methane oxidation rates *in situ*. Thus, the potential for nitrate-dependent AOM was demonstrated, but a quantitative assessment of the importance of AOM *in situ* demands further research.

The low rate of AOM in the absence of an external electron acceptor in the second littoral sediment sample might be a side effect of active methanogenesis in this sediment. Zehnder and Brock observed up to 8% label exchange during methanogenesis in Lake Mendota sediment (57). In our case, the measured formation of 380 nmol methane would be sufficient by far to explain the observed formation of up to 6 nmol $CO_2$. This kind of "AOM" is always linked to methane production and was presumably not taking place in any other treatment. Furthermore, nitrate is known to inhibit methanogenesis (29); thus, AOM in nitrate treatments is probably independent of methanogenesis.

AOM coupled to sulfate reduction was not detectable in any of our experiments. Furthermore, previous studies did not detect ANME archaea in sediments of Lake Constance by clone library analysis (42) or by fluorescence *in situ* hybridization (M. Rahalkar, personal communication). AOM coupled to sulfate reduction has been reported for various environments (30), including freshwater habitats (11, 36). So far, these reports were based on indirect evidence, and in most cases the possibility of involvement of a further electron acceptor besides sulfate cannot be excluded with certainty. Considering that in shallow freshwater systems the methane partial pressure can hardly rise far beyond 1 atmosphere and considering the low sulfate concentrations in limnic systems, the energy gain of sulfate-dependent AOM is most likely insufficient to fuel a syntrophic binary methane-oxidizing association in these environments.

An AOM coupled to iron or manganese reduction as recently described (2) was not investigated in our study but obviously did not take place at detectable rates, although ferric iron is present in the investigated sediment layers of Lake Constance (26). Also, the proposed coupling of AOM to the reduction of humic compounds (46) was not observed, although humic compounds are present in Lake Constance sediments (27).

**Presence of denitrifying anaerobic methanotrophs (NC10 bacteria).** The presence of NC10 bacteria was verified using molecular methods, and these bacteria might be responsible for AOM coupled to denitrification in our samples. NC10 bacteria appear to be widespread in Lake Constance, as specific 16S rRNA gene amplicons were obtained from DNA extracts taken at various locations in Lake Constance (data not shown). However, the community composition of NC10 bacteria appears to differ substantially between sites. The dominating clone sequences in profundal sediments form a very uniform cluster of group A NC10 bacteria, as grouped by Ettwig et al. (14), and appear to be absent or low in abundance in littoral sediment. Furthermore, 16S rRNA gene sequences that are almost identical to the dominant profundal cluster described in our study have been detected before in profundal sediments of Lake Washington and the mesotrophic Lake Biwa, where this 16S sequence (GenBank accession no. AB116934) is also present as rRNA at sediment depths down to 8 cm (31). Interestingly, by targeting the *pmoA* gene, PCR products were obtained only from profundal samples in which group A members were detected. Furthermore, the low diversity of *pmoA* gene sequences coincides well with the low diversity of group A NC10 bacteria on a 16S sequence basis, and both gene sequence clusters are similarly related to "*Candidatus* Methylomirabilis oxyfera" (3.4 to 6.6% on a 16S basis and 3.5% to 5.5% on a *pmoA* basis). Therefore, we hypothesize that only representatives of group A of the NC10 bacteria are responsible for nitrate-dependent AOM in Lake Constance. This would also explain the lower rates of nitrate-coupled AOM in littoral sediments where group A of the NC10 bacteria was present at levels below the detection limit. Additionally, only NC10 group A bacteria were enriched in various enrichments (14, 23, 41). However, it cannot be ruled out that our *pmoA* primers have a target range too narrow to amplify the entire diversity of *pmoA* genes affiliated with the NC10 phylum because there are almost no references available. Despite the indications that NC10 bacteria of group A are responsible for denitrifying AOM in Lake Constance, the involvement of other yet unknown organisms cannot be excluded.

Consistent data on denitrifying AOM rates and the presence of the respective bacteria were obtained for profundal sediments of Lake Constance, which provide constant environmental conditions, but not for the more disturbed littoral ones. The heterogeneity of littoral sediments was reflected in inconsistent data on denitrifying AOM rates, and no NC10 bacteria associated with denitrifying AOM have been detected. Thus, further research on the distribution of this process and the respective bacteria in heterogeneous environments and more extensive sampling might be required to allow general insights into their ecology.

Our study proves the presence of NC10 bacteria on the basis of 16S rRNA gene and *pmoA* sequence analysis in an oligotrophic environment with nitrate concentrations below 75 $\mu$M (39, 44) and shows that the recently discovered process of anaerobic methane oxidation coupled to denitrification can also take place in oligotrophic freshwater habitats like Lake Constance. Thus, we provide first evidence that this process might be widespread in freshwater habitats. However, further studies on other freshwater habitats have to follow to enable sound conclusions on the global importance of this methane sink which acts as a link between the carbon and the nitrogen cycle.

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